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Arch Virol (2006) 151: 1635–1641
DOI 10.1007/s00705-006-0739-6

Archives of Virology
Printed in Austria

Genetic analysis of noroviruses associated with fatalities in healthcare facilities

Brief Report

M. Okada[1], **T. Tanaka**[2], **M. Oseto**[3], **N. Takeda**[4], and **K. Shinozaki**[1]

[1]Division of Virology, Chiba Prefectural Institute of Public Health, Chiba, Japan
[2]Sakai City Institute of Public Health, Sakai, Osaka, Japan
[3]Microbiology Division, Ehime Prefecture Institute of Public Health and Environmental Science, Matsuyama, Ehime, Japan
[4]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

Received December 26, 2005; accepted January 26, 2006
Published online March 9, 2006

Summary. Norovirus outbreaks occurred in 236 healthcare facilities for the elderly in Japan during the winter of 2004–2005. Three norovirus strains associated with three fatal clinical courses were isolated from geographically separate facilities and genetically analyzed along with three strains from non-fatal cases in the same season. All six isolates were classified as the GII-4 genotype. No new variant strains like those observed in Europe in 2002 and 2004 were found in fatal cases, and the three outbreaks were deemed to have been caused by genetically close conventional norovirus GII-4 strains.

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Norovirus (NoV) is a leading cause of acute gastroenteritis in humans and animals [10, 14], causing worldwide outbreaks in various epidemiological settings including hospitals, nursing homes, schools and restaurants [4, 8, 9, 15]. Transmission of NoV occurs via the faecal-oral route, food-borne route, person-to-person contact, and environmental contamination, and infection occurs in all age groups [4, 8, 9]. Human NoV is divided into two genogroups, genogroup I (GI) and GII [1], which are further classified into 15 and 18 genotypes, respectively, based on the capsid protein [12]. The GII-4 genotype, represented by Lordsdale virus isolated in the United Kingdom in 1993 [13], is a dominant genotype worldwide [7, 11, 12].

Although NoV causes relatively mild gastroenteritis in healthy individuals [10] with few fatal cases, elderly and immunocompromised patients can suffer from severe gastroenteritis, sometimes resulting in death [2, 8]. Fatality rates

Fig. 1. Geographic relationships between three independent NoV outbreaks in Japan analyzed in this study. The geographic locations of the three outbreaks are shown in the map

associated with NoV outbreaks are reportedly 0.075 and 0.087% in England, Wales and the United States, respectively [8, 10]. In England and Wales, 43 fatal cases were observed in 38 outbreaks in hospitals and residential care facilities between 1992 and 2000. Recently, five fatal cases (fatality rate: 2.0%) associated with a large-scale gastroenteritis outbreak in nursing homes in Israel were also reported [2].

NoV outbreaks occurred in 236 healthcare facilities for the elderly in Japan in 2004–2005 with 12 fatal cases reported in six prefectures (http://www.mhlw.go.jp/houdou/2005/01/h0112-3.html). NoV samples were obtained from three independent fatal cases from geographically separate facilities in Chiba, Sakai and Ehime, respectively. The geographic locations of the three outbreaks analyzed in this study are shown in Fig. 1, and the epidemiological findings of the above three outbreaks are summarized in Table 1. Three NoV strains, Chiba/04-1050/2005 (Chiba/04-1050), Sakai/04-179/2005 (Sakai/04-179) and Ehime/05-30/2005 (Ehime/05-30), from these three fatal cases were analyzed. RNA extraction and RT-PCR targeting the 5′ end of open reading frame (ORF) 2 followed by genetic analysis were performed as described previously [12]. Comparisons of the nucleotide sequences demonstrated that these three strains had high nucleotide identities (approximately 99%), and these strains were classified into genotype GII-4 (data not shown). For further genome analysis, the NoV genome was amplified as three separate overlapping segments. The amplified products were directly sequenced as previously described [12], and the complete nucleotide sequence of Chiba/04-1050 and nearly complete nucleotide sequences minus the 5′ terminus of Sakai/04-179 and Ehime/05-30 were determined. Nucleotide sequences determined in this study were submitted to DDBJ with accession numbers AB220921 to AB220926.

Table 1. Summary of the three independent NoV outbreaks in healthcare facilities for the elderly analyzed in this study

	Case 1	Case 2	Case 3
Location Facility	Chiba Prefecture special nursing home for the elderly	Sakai City (Osaka Prefecture) hospital and healthcare facility for the elderly	Ehime Prefecture healthcare facility for the elderly
Total number of individuals			
residents	78	484 (in total)	97
workers	60		69
Affected individuals			
residents	43 (55.1%)	68 (14.0)%	35 (36.1)%
workers	20 (33.3%)		15 (21.7)%
Duration period	1st to 16th of January, 2005	3rd to 28th of January, 2005	2nd to 15th of January, 2005
Major symptoms	diarrhea, vomiting, fever, abdominal pain	diarrhea, vomiting, fever, abdominal pain	diarrhea, vomiting, fever
Number of death	1	1	1
Fatality rate	1.59%	1.47%	2.0%
NV testing methods	RT-PCR, electron microscopy	RT-PCR	RT-PCR, electron microscopy
Tested samples			
residents	10 stools & 1 vomitus	9 stools	12 stools & 2 vomitus
workers	3 stools	ND	2 stools
Positivity for NV[a]			
residents	7 [5] stools & 1[0] vomitus	7 stools	9 [6] & 2 [2] vomitus
workers	0 [0] stools	ND	2 [0] stools
Rate of positive samples[a]	57.1 [35.7]%	77.8%	81.3 [50.0]%
Enteric bacterial pathogen	not detected	not detected	not detected
Fatal cases			
age, sex	82 years, female	95 years, female	90 years, male
onset	8th January	9th January	7th January
death	10th January	17th January	10th January
Cause of death	suffocation as a result of vomiting	septicemia	Acute bleeding in the gastrointestinal tract
Sample ID	Chiba/04-1050/2005	Sakai/04-179/2005	Ehime/05-30/2005
Source of NV detection	stool on 9th January	stool on 17th January	vomitus on 10th January
NV genotype (ORF2)	GII-4	GII-4	GII-4

[a]Values in brackets show positivity with electron microscopy; other value show positivity with RT-PCR

Chiba/04-1050 was composed of 7,559 nucleotides without a poly-A tail, while the other two strains from Sakai and Ehime comprised 7,533 nucleotides lacking the 5′ terminus. The average nucleotide identities among the three strains were 99.2% in ORF 1, 98.6% in ORF 2, and 98.8% in ORF 3. In addition, 10, 4

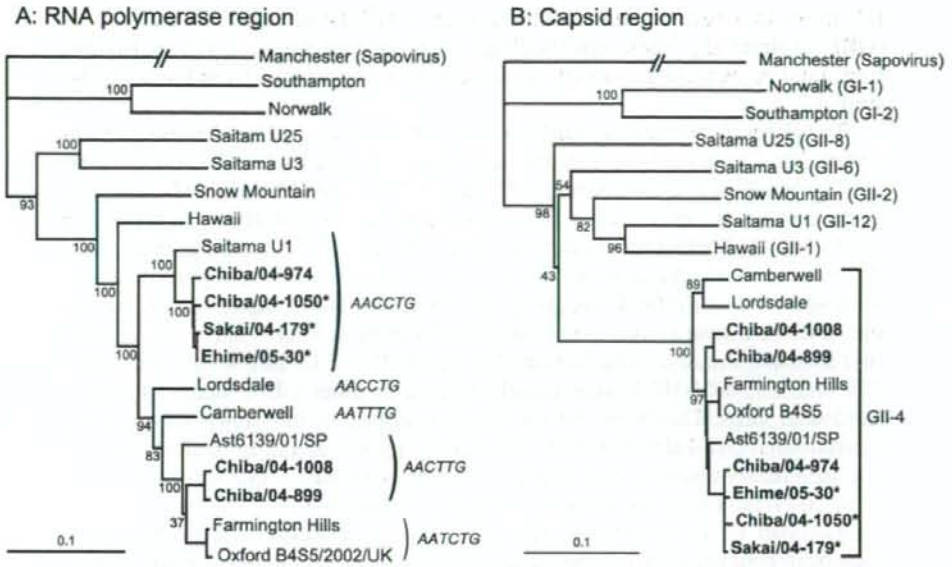

Fig. 2. Phylogenetic trees were constructed using the neighbor-joining method based on part of the RdRp region corresponding to 4307–5017 (**A**) and the capsid region corresponding to 5085–5509 (**B**) of Lordsdale virus. A sapovirus, Manchester strain, was used as the out-group. The six strains examined are shown in bold. Three strains from fatal cases are shown by asterisks, in which the complete and nearly complete genomes were amplified by RT-PCR with the following primers: NV5END (GAATGAAGATG GCGTCTAACGACG) and NV2690R (TGAGACCTTTGCTTGAGAAGGCTGT) for the 5′ genome region, NV2570F (CCAAAACCCAAAGATGATGAGGAGT) and NV5550R (GGTAAGGGGATCAACACAGGTTCCA) for the central region, and G2F1 and dT25VN [(T)25V(A/G/C)N(A/G/C/T)] for the 3′ genome region [12]. The 5′ end of the genome was amplified with the 5′ RACE Amplification System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Nucleotide sequences characterized as GII-4 variants reported by Lopman et al. [7] are also indicated in italic on the tree (A). Reference strains were Manchester virus (X86560), Norwalk virus (M87661), Southampton virus (L07418), Snow Mountain virus (AY134748), Lordsdale virus (X86557), Camberwell virus (AF145896), Farmington Hills (AY502023), Ast6139/01/SP (AJ583672), Oxford B4S5 (AY587984), Saitama U25 (AB067543), SaitamaU3 (AB039776) and Saitama U1 (AB039775)

and 6 amino acid substitutions were identified in each ORF. Phylogenetic trees based on the partial RNA-dependent RNA polymerase (RdRp) region (ORF1) and partial capsid region (ORF2) are shown in Fig. 2A and B. Phylogenetic analysis based on ORF2 indicated that the three strains were genetically close and clustered together with known GII-4 strains (Fig. 2B). These strains were also clustered with GII-4 strains when the RdRp region was compared (Fig. 2A). The Saitama

U1 strain is a recombinant strain between a GII-4-like (ORF 1) and GII-12 (ORF 2) strain [5]. These results clearly indicate that the three strains isolated from fatal cases were genetically close and indistinguishable from known GII-4 strains.

To further investigate the RdRp and capsid regions, an additional three strains detected in Chiba prefecture in the same season, Chiba/04-899/2004 (Chiba/04-899; outbreak in a nursery school), Chiba/04-974/2004 (Chiba/04-974, sporadic gastroenteritis patient) and Chiba/04-1008/2004 (Chiba/04-1008, outbreak in a healthcare facility) were similarly analyzed. Based on the capsid protein, these three strains were also grouped into GII-4 and shown to be closely related to the three strains from the fatal cases (Fig. 2B). When the RdRp region was compared, the three fatal case strains and Chiba/04-974 were closely related and grouped into a cluster including the Saitama U1 strain (Fig. 2A). In contrast, Chiba/04-899 and Chiba/04-1008 were closely related to other GII-4 strains including Lordsdale virus. Therefore, the six strains analyzed in this study were deemed conventional GII-4 strains widely circulating in this season. In addition, at least two genetically distinct GII-4 strains with different ORF1 sequences were shown to be co-circulating at the same time in Chiba prefecture.

Lopman et al. reported an increase in NoV-associated gastroenteritis in European countries due to emergence of new genetic variants of the GII-4 strain [7]. GII-4 strains detected before 2002 have an "AACTTG" sequence in the RdRp region while those detected in 2002 (new variants) show "AATCTG" [7]. Intermediate sequences have also been observed [7, 13]. Of the six GII-4 strains analyzed in this study, the three fatal strains and Chiba/040974 showed an intermediate sequence, "AACCTG" (Fig. 2A). The other two strains, Chiba/040899 and Chiba/041008, had 10 nucleotide substitutions in the RdRp region, which were observed in GII-4 2004 variant strains [6]. Therefore, no new variant strains like those isolated in 2002 and 2004 were identified in fatal cases in this study.

Previous studies have described the GII-4 genotype as the dominant genotype of NoV-associated gastroenteritis worldwide [7, 12, 15]. Furthermore, GII-4 strains are mainly detected in outbreaks in healthcare facilities such as nursing homes and hospitals [4, 9]. Lopman et al. reported that outbreaks in healthcare facilities showed a higher death rate and prolonged duration when compared to other outbreak settings [8]. Recently, fatal cases associated with GII-4 NoV outbreaks in nursing homes have been reported in Israel [2]. We detected genetically similar GII-4 strains from three independent outbreaks in geographically isolated healthcare facilities in Japan. Sequence analysis comparisons with an additional three strains from Chiba prefecture clearly indicated that these strains were not specific to these outbreaks. Although NoV infection is not likely the principal cause of death in most cases, NoV-associated outbreaks occurring in healthcare facilities for the elderly might constitute an additional burden. As neither common food nor food stuff was identified in the three fatal cases presented here, person-to-person transmission by either direct contact with stool or vomitus or through the caregiver was considered the most likely mode of transmission. These findings

suggest that we need to pay more attention to the activity of NoV, especially that of the GII-4 genotype, in outbreaks in healthcare facilities.

Acknowledgment

This work was supported in part by a grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare, Japan.

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Author's address: Mineyuki Okada, Division of Virology, Chiba Prefectural Institute of Public Health, 666-2, Nitona-Cho, Chuo-Ku, Chiba-Shi, Chiba 260-8715, Japan; e-mail: m.okd4@mc.pref.chiba.jp

Journal of Medical Virology 78:971–978 (2006)

Changing Distribution of Norovirus Genotypes and Genetic Analysis of Recombinant GIIb Among Infants and Children With Diarrhea in Japan

Tung Gia Phan,[1] Toshimasa Kuroiwa,[1] Kunio Kaneshi,[1] Yuichi Ueda,[1] Shigekazu Nakaya,[1] Shuichi Nishimura,[1] Atsuko Yamamoto,[1] Kumiko Sugita,[2] Tadashi Nishimura,[2] Fumihiro Yagyu,[1] Shoko Okitsu,[1] Werner E.G. Müller,[3] Niwat Maneekarn,[4] and Hiroshi Ushijima[1]*

[1]*Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*
[2]*Department of Pediatrics, Hokusetsu Hospital, Osaka, Japan*
[3]*Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität, Mainz, Germany*
[4]*Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*

A total of 402 fecal specimens collected during July 2003–June 2004 from infants and children with acute gastroenteritis, encompassing five localities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) of Japan, were tested for the presence of norovirus by RT-PCR. It was found that 58 (14.4%) fecal specimens were positive for norovirus. Norovirus infection was detected throughout the year with the highest prevalence in December. Norovirus GII was the most predominant genogroup (98.3%; 57 of 58). The genotypes detected in this study were GI/4, GII/2, GII/3, GII/4, and GII/6. Of these, NoV GII/3 (known as the Arg320 virus cluster) was the most predominant genotype (43.9%), followed by NoV GII/4 (the Lordsdale virus cluster; 35.1%) and others. Two norovirus strains clustered with a "new variant designated GIIb" and a "new variant of GII/4" were found circulating in Japan for the first time. It was interesting to note that NoV GIIb and NoV GII/3 appeared to be the recombinant strains and the recombination site was demonstrated at the overlap of ORF1 and ORF2. The majority (96%) of the dominant norovirus strains were identified as the recombination of GII/3 capsid and GII/12 polymerase. The recombination in the NoV GIIb capsid gene at the breakpoint located at P1 domain was also identified. Obviously, NoV GIIb isolate in Japan had double recombination. This is the first report demonstrating the existence of different "new variants" co-circulating in Japanese infants and children with acute gastroenteritis. ***J. Med. Virol. 78:971–978, 2006.***

KEY WORDS: PCR; norovirus; recombination; Japan

INTRODUCTION

Norovirus (NoV) is recognized as a significant global enteropathogen, being a major cause of sporadic cases as well as of outbreaks of acute gastroenteritis in humans in various epidemiological settings, such as restaurants, schools, day-care centers, hospitals, nursing homes, and cruise ships [Chiba et al., 1979; McEvoy et al., 1996; Vinje et al., 1997; McIntyre et al., 2000]. The virus can be transmitted by food-borne, water-borne, air-borne, person-to-person spread by close contact and there might be some other unknown modes [Matson, 1994; Bon et al., 1999; Marks et al., 2000; Lopman et al., 2002; Oh et al., 2003]. NoV is highly infectious and spreads by ingestion of contaminated food such as oysters and water. These characteristics make NoV a major public health concern [Kageyama et al., 2004]. NoV is the distinct genus within the family *Caliciviridae*. The prototype strain of NoV is the Norwalk virus (Hu/NoV/Norwalk virus/1968/US), which was originally discovered from an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio, USA in 1968. NoV possesses a positive sense single-strand RNA genome surrounded by an icosahedral capsid. The NoV genome contains three open reading frames (ORFs). ORF1

Grant sponsor: Ministry of Education and Sciences and the Ministry of Health, Labor and Welfare, Japan; Grant sponsor: Heiwa Nakajima Foundation, the Mishima-Kaiun Foundation; Grant sponsor: Sumitomo Foundation in Japan.

*Correspondence to: Hiroshi Ushijima, Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
E-mail: ushijima@m.u-tokyo.ac.jp

Accepted 29 January 2005
DOI 10.1002/jmv.20649
Published online in Wiley InterScience (www.interscience.wiley.com)

encodes the non-structural proteins, including the RNA-dependent RNA polymerase (RdRp) while ORF 2 encodes the capsid protein (VP1) and ORF3 encodes a small capsid protein (VP2). Based on the sequence analysis of the capsid gene, NoV is divided into genogroups I and II, both known to infect humans. A recent study indicated that NoV GI and NoV GII could be classified into 14 and 17 genotypes, respectively [Kageyama et al., 2004]. The first naturally occurring recombinant NoV was the prototype Snow Mountain virus [Hardy et al., 1997]. Later several recombinant NoV strains causing sporadic cases and outbreaks of acute gastroenteritis were reported [Jiang et al., 1999a; Schreier et al., 2000; Katayama et al., 2002]. RNA recombination is one of the major driving forces of viral evolution [Worobey and Holmes, 1999]. To date, NoV is still uncultivable by standard culture methods with different cell lines. However, either VP1 alone or both VP1 and VP 2 could be expressed using recombinant baculovirus forming virus-like particles (VLPs) that are similar morphologically and antigenically to the native virion [Jiang et al., 1995]. Seroepidemiologic studies indicated a worldwide distribution of NoV. Moreover, it was found that serum antibody level to NoV was lowest in the first year of life and then rising after 2 years of age [Lopman et al., 2002; Dai et al., 2004; Peasey et al., 2004].

The objectives of this study were: to determine the incidence of NoV infections in infants and children with acute gastroenteritis in five different localities of Japan during 2003 and 2004; to characterize the genogroup and genotype of the detected NoV; and to describe the genetic diversity among them. Additionally, the age-related and seasonal distributions of NoV infection were determined.

MATERIALS AND METHODS

Fecal Specimens

A total of 402 fecal specimens were collected from infants and children with acute gastroenteritis, encompassing five different localities (Maizuru, Tokyo, Sapporo, Saga, and Osaka) of Japan during the period of July 2003–June 2004. Of these, 19 specimens were from Osaka, 22 from Sapporo, 22 from Tokyo, 45 from Saga, and 294 from Maizuru. The ages of the subjects were ranged from 2 months to 11 years with the median of 2.5 years (29 months). The majority (75%) of the affected children were aged less than 36 months and about half (54%) were male. The 10% fecal suspension was prepared in distilled water and clarified by centrifugation at 10,000*g* for 10 min. The supernatant was collected and stored at −30 C until use.

Extraction of Viral Genome

The viral genomes were extracted from 140 μl of 10% fecal suspensions using the QIAamp viral RNA Mini Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions.

Reverse Transcription (RT)

For reverse transcription (RT), 7.5 μl of extracted viral genome was added to the reaction mixture containing 2.05 μl of 5× first strand buffer (Invitrogen, Carlsbad, CA), 0.75 μl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.75 μl of 10 mM DTT (Invitrogen), 0.75 μl (200 U/μl) of superscript reverse transcriptase III (Invitrogen), 0.375 μl (1 μg/μl) of random primer (hexadeoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 μl (33 U/μl) of RNase inhibitor (Toyobo, Osaka, Japan), and 2.325 μl MilliQ water. The total volume of reaction mixture was 15 μl [Yan et al., 2003]. The RT step was carried out at 50 C for 1 hr, followed by 99 C for 5 min and then held at 4 C.

Polymerase Chain Reaction (PCR)

The NoV genogroups were identified by PCR method using specific primers as described [Yan et al., 2003]. Two pairs of specific primers G1SKF (CTGCCCGAATTYGTAAATGA) and G1SKR (CCAACCCARCCATTRTACA), and COG2F (CARGARBCNATGTTYAGRTGGATGAG) and G2SKR (CCRCCNGCATRHCCRTTRTACAT) [where B was C, G or T; H was A, C or T; N was any base; R was A or G, and Y was C or T] that amplify capsid gene of NoV were used to detect NoV GI and NoV GII, respectively. These primers were specifically generated two different sizes of amplicons of 330 and 387 bp for NoV GI and NoV GII, respectively. The RNA polymerase gene of NoV was also amplified to identify the recombinant strain of NoV using the primers as described [Jiang et al., 1999b; White et al., 2002]. The PCR was carried out with 2.5 μl of cDNA in 22.5 μl of the reaction mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI), dNTPs (2.5 mM/μl), primers (33 μM), Taq DNA polymerase (5 U/μl) (Promega, Madison, WI) and MilliQ water. The PCR was performed at 94 C for 3 min followed by 35 cycles of 94 C for 30 sec, 55 C for 30 sec, 72 C for 60 sec, and a final extension at 72 C for 7 min, and then held at 4 C. The full length of capsid and polymerase regions were amplified with a newly designed specific primer NVPOLR/A (GAT GAG GTT CTG ATG AGA) and the specific primers reported by Vinje et al. [2000] and Kawamoto et al. [2001]. The PCR was performed at 94 C for 3 min followed by 35 cycles of 94 C for 1 min, 55 C for 2 min, 72 C for 3 min, and a final extension at 72 C for 7 min, and then held at 4 C.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min and then visualized under ultraviolet (UV) light. The results were recorded by photography.

Nucleotide Sequencing and Phylogenetic Analysis

The nucleotide sequences of PCR products (DNA) positive for NoV were determined using the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc., Foster

City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.6). Phylogenetic tree with 100 bootstrap resamples of the nucleotide sequence alignment data sets were generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). SimPlot software (Version 1.3) was used to compare the recombinant NoV sequences [Lole et al., 1999]. The nucleotide sequences of NoV strains 5424/03/Saga/JP and 5017/04/Maizuru/JP had been submitted to the DDBJ DNA/GenBank database and the assigned accession numbers were AB242256 and AB242257, respectively. Reference NoV strains and accession numbers used in this study were as follows: Manchester (X86560), Saitama T53GII/02/JP (AB112260), Girllington (AJ277606), Melksham (X81879), Chitta (AB032758), Wortley (AJ277618), Hillington (AJ277607), Alphatron (AF195847), Toronto (U02030), Seacroft (AJ277620), Leeds (AJ277608), Lordsdale (X86557), Idaho Falls/96/US (AY054299), Fayetteville/1998/US (AY113106), Erfurt/546/00/DE (AF42118), M7/99/US (AY130761), Saitama U1 (AB039775), Camberwell (AF145896), Snow Mountain (U70059), Paris Island/2003/USA (AY652979), Oberhausen 455/01/DE (AF539440), C14/2002/AU (AY845056), Herzberg 385/01/DE (AF539439), Arg320 (AF190817), VannesL169/2000/France (AY773210), Amsterdam (AF195848), White River/94/US (AF414423), Mexico (U22498), MD145 (AY032605), Mora/97/SE (AY081134), SaitamaT29GII/01/JP (AB112221), SaitamaKU80aGII/99/JP (AB058582), Mc37 (AY237415), Stockholm/IV4348/01/SE (AJ626633), and Pont de Roide 673/04/France (AY682549).

RESULTS

Epidemiology of Norovirus Infections

A total of 402 fecal specimens collected from infants and children with acute gastroenteritis from five different localities of Japan during July 2003 and June 2004 were examined for the presence of NoV. NoV was detected in 58 out of 402 (14.4%) specimens tested. The highest prevalence of NoV was found in infants and children with the age range of 12–23 months (36.2%). No case of NoV infection was identified among infants aged less than 6 months. It was also found that infants and children younger than 3 years old had a high rate of NoV infection (79.3%). NoV was detected throughout the period of 9 months starting from October 2003 to June 2004. However, none of NoV was detected from July to September 2003. The NoV incidence was found highest in December (27.5%), followed by November (19%), and January (12.1%). The lowest NoV detection rate fell into October (5.2%).

Nucleotide Sequence and Phylogenetic Analyses of NoV Genotypes

The partial nucleotide sequences of capsid gene of NoV detected in this study were compared to each other as well as to those of NoV reference strains available in the DDBJ DNA/GenBank database by BLAST. A total of 58 NoV nucleotide sequences, including 1 of NoV GI and 57 of NoV GII were analyzed by phylogenetic grouping based on the recent NoV capsid region classification schemes described by Kageyama et al. [2004]. It was found that the NoV GI sequence (5226/04/Maizuru/JP) clustered into one distinct group with GI/4, which was represented by the Chiba/87/JP virus cluster. It was of interest to note that the 5226/04/Maizuru/JP strain showed the genetic relationship with the NoV Mie2001-U94/JP, which was previously isolated from oyster in Japan, with the nucleotide sequence identity to as high as 98%. Additionally, the 5226/04/Maizuru/JP strain revealed 90%–97% nucleotide sequence identities with those of other human NoV reference strains.

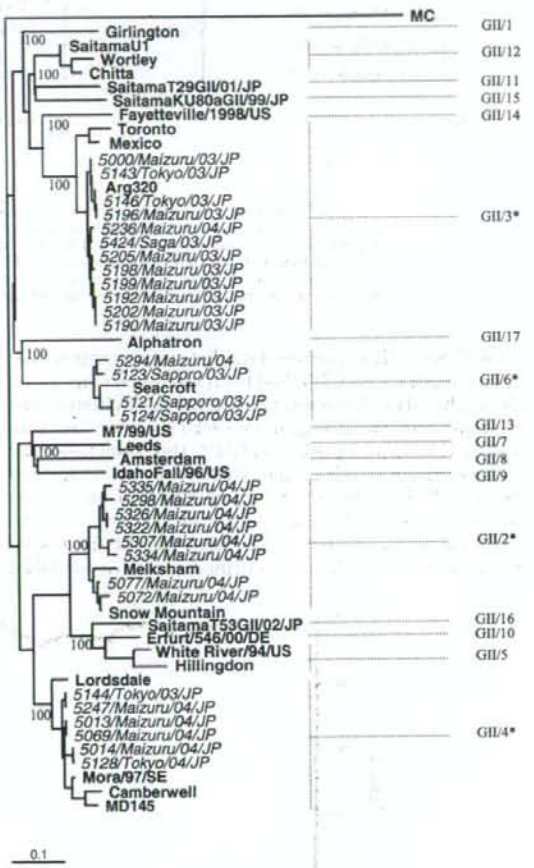

Fig. 1. Phylogenetic tree of nucleotide sequences of Japanese NoV GII. The tree was constructed from partial nucleotide sequences of capsid region of NoV GII isolates detected in Japan. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Japanese NoV was highlighted in italic. MC strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values if 100% is given. *, Genotype contains Japanese NoV detected in the study.

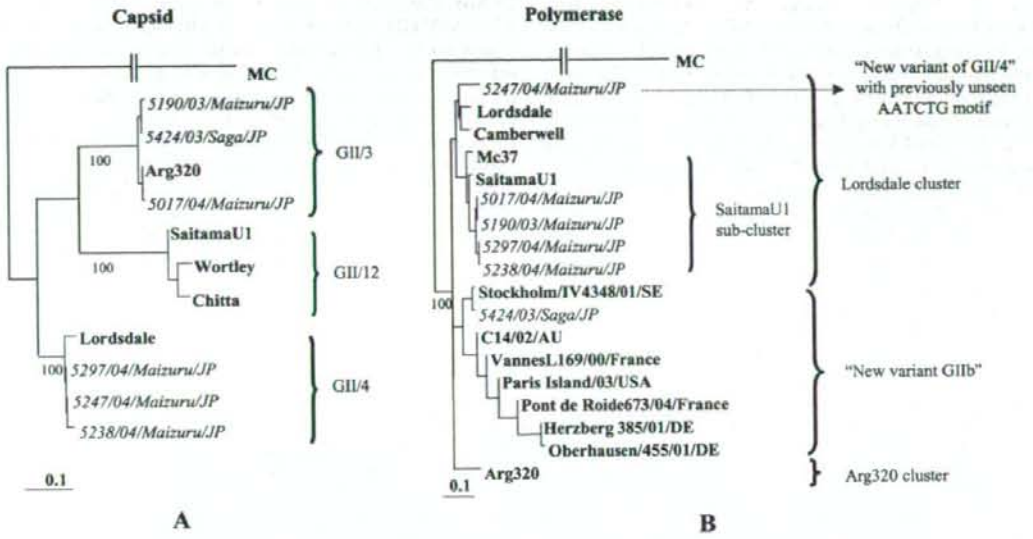

Fig. 2. Observation of changes of NoV genotypes on the basis of phylogenetic trees of nucleotide sequences. The trees were constructed from partial nucleotide sequences of capsid and polymerase regions of the Japanese representative isolates of NoV GII. Reference strains of NoV were selected from the DDBJ DNA/GenBank database under the accession numbers indicated in the text. Japanese NoV was highlighted in italic. MC strain was used as an out-group strain for phylogenetic analysis. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values if 100% is given.

Of 57 NoV GII sequences, four distinct genotypes, GII/2, GII/3, GII4, and GII/6 had been identified (Fig. 1). Of these, the GII/3 (known as the Arg320 virus cluster) was the most predominant genotype with the prevalent rate of 43.9%, followed by 35.1% of GII/4 (the Lordsdale virus cluster), 14% of GII/2 (the Melksham virus cluster), and 7% of GII/6 (the Seacroft virus cluster). Considering the genotype distribution by localities, GII/3 was also the most predominant in all localities, except for Osaka where none of GII/3 was identified and only one GII/4 was detected in Osaka. The nucleotide sequence identities were ranged from 58% to 99% when NoV GII strains detected in this study were compared with those the reference strains previously registered in the DDBJ DNA/GenBank database.

Nucleotide Sequence and Genetic Analyses of NoV RNA Polymerase Gene

To verify the changing epidemiology of NoV genotypes, the RNA polymerase genes of all NoV GII/3 and

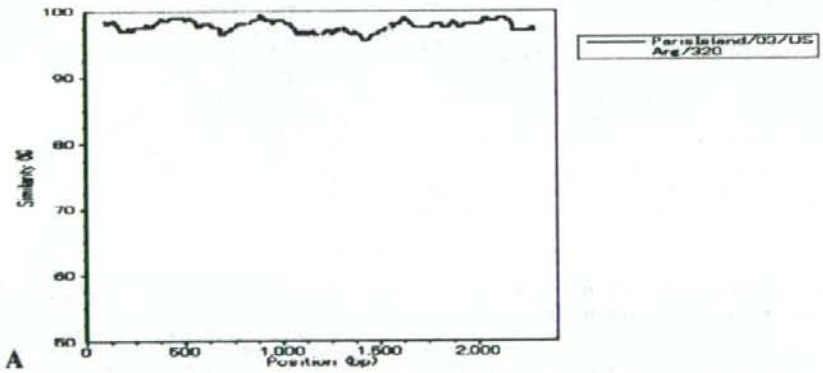

Fig. 3. Genetic characterization of recombinant NoV "new variant with polymerase GIIb." **A**: The Simplot analysis of the 5424/03/Saga/JP, the Paris Island/03/USA, and the Arg320. **B**: Evidence of recombination in NoV capsid gene.

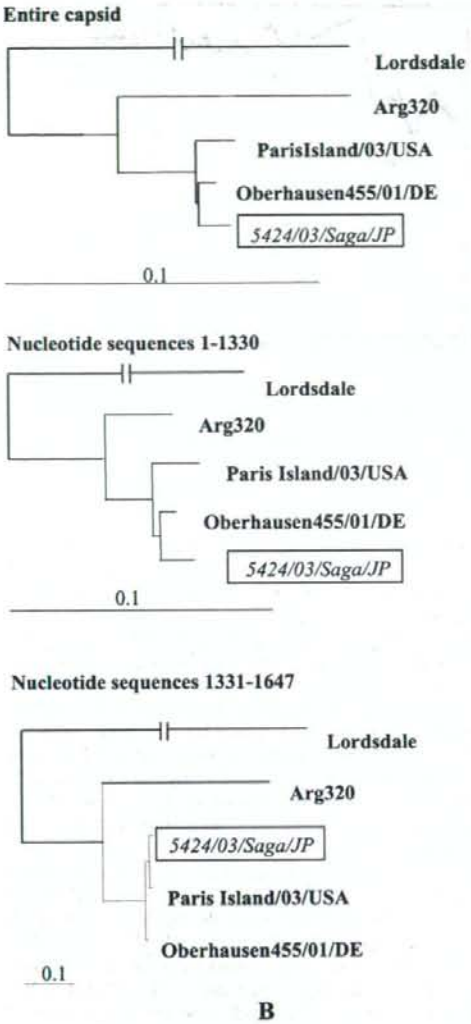

Fig. 3. (*Continued*)

NoV GII/4 were additionally amplified and sequenced. Of 25 NoV isolates with GII/3 capsid, 24 were classified into the SaitamaU1 sub-cluster (known as GII/12) but not into the Arg320 cluster when polymerase-based grouping was performed. The findings suggested that these 24 isolates were all recombinant viruses with GII/3 capsid and GII/12 polymerase. Interestingly, another NoV isolate, the 5424/03/Saga/JP, was grouped with NoV reference "new variants," which were designated as a GIIb in European countries (Fig. 2B). Taken together, the results indicated that the 5424/03/Saga/JP was also the recombinant strain.

In contrast, 20 NoV isolates belonging to GII/4 (the Lordsdale virus cluster), the genotype remained the same no matter the polymerase or capsid regions, was analyzed. Of these, 19 isolates shared significantly high identity of polymerase nucleotide sequences ranging from 98% to 100%. However, they shared only 93% sequence identity with those of the 5247/04/Maizuru/JP. It should be noted that the 5247/04/Maizuru/JP contained the previously unseen AATCTG motif starting at position 4,820 in the polymerase region (referring to the Norwalk virus, M87661). Obviously, this isolate was recognized as a "new variant of GII/4" according to the definition of Lopman et al. [2004]. Furthermore, as shown in Figure 2A, the majority (77.6%, 45 of 58) of NoV isolates were classified into GII/3 and GII/4 based on the partial capsid region, however, they were grouped into a SaitamaU1 sub-cluster based on the partial polymerase region (Fig. 2B).

Genetic Characterization of Recombinant Strain With GIIb Polymerase

To localize the potential recombination site and to understand a possible recombination mechanism of the "new variant" GIIb, the full-length nucleotide sequences of capsid and polymerase regions were determined and analyzed. When the nucleotide sequence of the 5424/03/Saga/JP was compared with those of the Arg320 and the Paris Island/03/US using the SimPlot software, region of genetic recombination was found between nucleotides 1,514 and 1,533 (the overlap of ORF1 and ORF2) (Fig. 3A). Up stream to this junction the nucleotide homology was notably different, and the SimPlot analysis showed a sudden drop in the nucleotide identity for the Arg320 but not for the 5424/03/Saga/JP and the Paris Island/03/US. The results demonstrated that the nucleotide sequences of capsid genes among these three strains were almost identical, but the polymerase sequences of the 5424/03/Saga/JP and the Paris Island/03/US were distinctly different from that of the Arg320.

Within the 5424/03/Saga/JP capsid sequence, the recombination at the breakpoint located at the beginning of P1 domain (position 1,330 nt in the capsid region) was identified. The capsid sequence of the recombinant 5424/03/Saga/JP showed alternate identities to the Oberhausen455/01/DE (nucleotides 1–1,330) and the Paris Island/03/USA (nucleotides 1,331–1,647) (Fig. 3B). The Oberhausen455/01/DE was detected in 2001 in Germany, whereas the Paris Island/03/USA was detected in 2003 in the United States. Quite possibly, the Paris Island/03/USA and the Oberhausen455/01/DE were putative parental strains of the 5424/03/Saga/JP. Taken together, the findings indicated that the 5424/03/Saga/JP had a double recombination.

Genetic Characterization of Recombinant Strain With SaitamaU1 Polymerase

As mentioned above, 24 isolates of GII/3 had high homology (98%–100%) at the nucleotide level of capsid

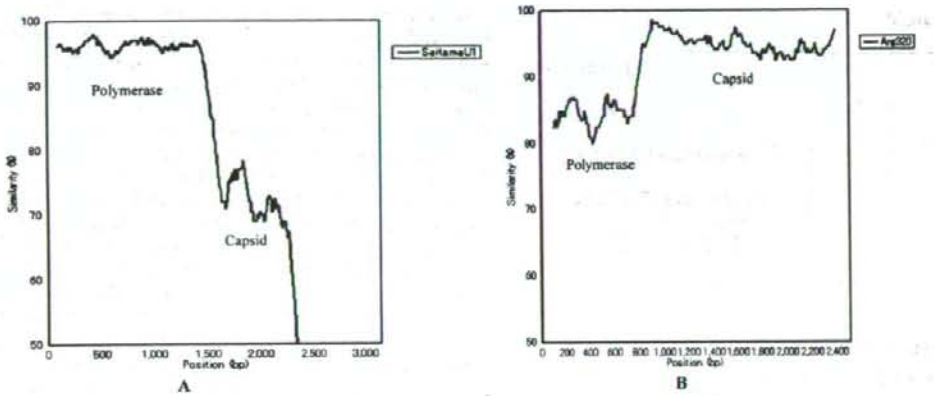

Fig. 4. Genetic characterization of recombinant virus with GII/3 capsid. **A**: The Simplot analysis of the NoV representative isolate, the 5017/04/Maizuru/JP, and the reference strain SaitamaU1. The high and low homologies with the polymerase and capsid regions among them, respectively, were found. **B**: The Simplot analysis of the 5017/04/Maizuru/JP and the reference strain Arg320. The low and high homologies with the polymerase and capsid regions among them, respectively, were found.

and polymerase. The findings demonstrated that they came from the same source of infection and represented the same strain. Furthermore, they were also suspected to be recombinant NoV based on their partial capsid and polymerase sequence. To further analyze this finding, the complete nucleotide sequences of the capsid and polymerase regions of one representative isolate, the 5017/04/Maizuru/JP, were determined. The 5017/04/Maizuru/JP shared a consistently low level of sequence identity (84%) in the RNA polymerase region but consistently high identity (95%) in the capsid region with the Arg320. In contrast, the 5017/04/Maizuru/JP shared consistently high level of sequence identity (96%) in the polymerase region and consistently low identity (70%) in the capsid region with those of the SaitamaU1. A recombinant site was also observed at the overlap of ORF1 and ORF2 (Fig. 4).

DISCUSSION

In this study, the prevalence of NoV infection among infants and children with acute gastroenteritis in five different localities of Japan was reported. Overall, the prevalence rate was 14.4% in all age groups of the subjects included in this study. However, the prevalence rate was increased up to 79.3% in infants and young children with the ages of less than 3 years old. These results were consistent with previous reports on NoV epidemiology worldwide in which the prevalence was ranged from 10% to 60% or more [Marks et al., 2000; Iritani et al., 2002; Lopman et al., 2002; Oh et al., 2003; Phan et al., 2004]. The findings suggested that approximately 14.4% of the etiologic agents of acute gastroenteritis cases in infants and children in Japan might be due to NoV and 85.6% might be responsible by other pathogens. The result also confirmed that NoV is one of the important enteropathogens responsible for viral gastroenteritis among infants and children in Japan. In some reports, NoV was prevalent in cold season, and several studies did not find a seasonal correlation [Vinje et al., 1997; Lopman et al., 2002; Phan et al., 2004]. The findings in this study are in agreement with the surveillance on pediatric cases of viral gastroenteritis in Japan, which demonstrated that the main peak of NoV infection was in the period of November, December, and January [Iritani et al., 2003; Inouye et al., 2000].

The results of this study showed that all Japanese NoV isolates belonged to two distinct genogroups, GI and GII, and these represented 1.7% and 98.3%, respectively. The results indicated that NoV GII was the dominant group causing acute gastroenteritis among Japanese pediatric population. It was interesting to note that the NoV GI 5226/04/Maizuru/JP, which was recovered from one 6-year-old-female patient with diarrhea, was closer genetically to the NoV Mie2001-U94/JP isolated from Japanese oyster than to human NoV reference strains available in the DDBJ DNA/GenBank database. This finding supported a view of possible NoV transmission to human through the contaminated oyster, which known as a reservoir of NoV. According to other reports published by different groups of investigators, NoV belonging to the Lordsdale cluster (GII/4) represented the most predominant genotype detected in sporadic gastroenteritis among infants and children not only in Japan but also in many other countries who run NoV surveillance [Chiba et al., 1979; McEvoy et al., 1996; Vinje et al., 1997; McIntyre et al., 2000]. However, it is surprising to note that in the present study NoV GII/3 was the most predominant, followed by NoV GII/4, NoV GII/2, and NoV GII/6. To verify this unusual observation, the polymerase regions of all NoV GII/3 and NoV GII/4 were further characterized. Remarkably, all NoV GII/3 except the 5424/04/Saga/JP were identified as the recombinant viruses that

related genetically to the SaitamaU1-like polymerase and the Arg320-like capsid. More interestingly, the SaitamaU1-like polymerase of NoV GII/3 was identical with those of NoV GII/4. The recombination of the NoV GII/4 polymerase and the Arg320-like capsid leading to an appearance of novel recombinant virus in the present study is postulated. Recently, NoV capsid protein was demonstrated to contain the determinants that are important for the immune recognition [Nilsson et al., 2003; Kirkwood, 2004]. Therefore, the emergence of recombinant virus with GII/3 capsid could be explained by a lack of acquired immunity for NoV GII/3 in Japanese infants and children. Interestingly, these recombinant strains suddenly appeared in a short period of 4 months (October 2003–January 2004) (data not shown). This sudden appearance and disappearance of strains might indicate that the virus appeared at the time that pediatric population lack antibody protection to these strains, and the virus disappeared by the time that the population began to acquire viral immunity. However, several studies reported that dominant strains could persist in one region over a number of years, which suggests that some other uncommon strains could be more virulent [Noel et al., 1999; Phan et al., 2004].

Another interesting finding of this study was the detection of "new variant with GIIb polymerase" 5424/03/Saga/JP in Japan. This isolate was isolated from a male patient with the age of 2 years old who developed a symptom of acute gastroenteritis in Saga, Japan. Surprisingly, based on the genetic analysis, this strain appeared to be an intratypic double recombinant. More interestingly, "new variant of GII/4 with unseen AATCTG motif" was also detected for the first time in a 2-year-old male patient with acute gastroenteritis in Maizuru, Japan in 2004. This motif was not present in any of the GII/4 sequences analyzed worldwide before 2002 from the food-borne viruses in European database and from the DDBJ DNA/GenBank database. This variant was first noted in Germany and the Netherlands in 2002 and become the predominant cause of NoV outbreaks throughout Europe [Lopman et al., 2004].

In conclusion, this is the first report on the existence of different "new variants" co-circulating in Japanese infants and children with acute gastroenteritis. This is also the first, description to the best of our knowledge, of the emergence and the importance of a novel recombinant virus causing acute gastroenteritis in Japan and warns of the threat it poses. Further epidemiologic studies should be conducted to determine whether this recombinant strain continues to be dominant in Japan in the coming year.

ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid from the Ministry of Education and Sciences and the Ministry of Health, Labor and Welfare, Japan. This study was also supported by the Heiwa Nakajima Foundation, the Mishima-Kaiun Foundation, and the Sumitomo Foundation in Japan.

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Journal of Medical Virology 78:1318–1324 (2006)

Existence of Multiple Genotypes Associated With Acute Gastroenteritis During 6-Year Survey of Norovirus Infection in Japan

Michio Okame, Shiho Akihara, Grant Hansman, Yan Hainian, Huy Thien Tuan Tran, Tung Gia Phan, Fumihiro Yagyu, Shoko Okitsu, and Hiroshi Ushijima*

Department of Developmental Medical Sciences, Institute of International Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Norovirus (NoV) is recognized as one of the most common causative agent of diarrheal disease in young children worldwide. The current study was undertaken to determine the distribution of NoV genotypes in Japan. A total of 3,864 fecal specimens from children with acute gastroenteritis in five regions (Tokyo, Maizuru, Saga, Sapporo, and Osaka) of Japan from July 1995 to June 2001 were collected and then tested for the presence of NoV by RT-PCR. Three hundred sixty four were found to be positive for NoV, accounting for 11%. The highest prevalence of NoV infection was in November, December, and January as the early winter months in Japan. NoV was subjected to be further characterized to sequencing analysis. All NoVs belonged to two different genogroups I and II and these represented 3% and 97%, respectively. This finding indicated that NoV genogroup II was the dominant group causing acute gastroenteritis in Japan. Interestingly, NoV strains were classified into 16 distinct genotypes including genogroup II genotype 9 that was firstly identified in Japan. Of these, NoV genogroup II genotypes 3 and 4 dominated over other genotypes and became the leading strains in Japanese pediatric population. In conclusion, diarrhea due to NoV infection is still a health burden in Japan. This report also stresses the great genetic diversity as well as the importance of NoV causing the diarrhea in Japan. ***J. Med. Virol. 78:1318–1324, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: norovirus; genotype; diversity; Japan

INTRODUCTION

Norovirus (NoV) is in the family *Caliciviridae* and contains a single-stranded positive-sense RNA genome, approximately 7.7 kb in size. The NoV genome composes of three open reading frames (ORFs). ORF1 encodes non-structural proteins, including the RNA-dependent RNA polymerase, ORF 2 encodes the capsid protein, and ORF3 a small capsid protein. To date, NoV can be genetically divided into three genogroups (GI, GII, and GIII) based on genome sequence. Of these, NoV GI and GII are known to infect humans and NoV GIII infects animals including bovine and murine. NoV cannot be cultivated in cell culture or experimental animal models. Detection of NoV has relied mainly on RT-PCR using specific primers with the binding sites at the polymerase region or the capsid region [Katayama et al., 2002]. For the genetic classification of NoV, the polymerase region or the capsid region has been used independently. Recently, genetic classification of NoV has described at least 14 and 17 different genotypes for NoV GI and GII, respectively [Kageyama et al., 2004] in which strain Alphatron belongs to NoV genogroup II genotype 17. This capsid region-based classification appeared to distinguish successfully the antigenicity determined by both antigen and antibody ELISA with recombinant virus-like particle [Kobayashi et al., 2000a,b]. Hardy et al. [1997] reported a naturally, occurring recombinant in NoV, then several NoV strains have been described as recombinants and the recombination site were found at the junction of ORF1 and ORF2 [Jiang et al., 1999; Hansman et al., 2004].

Norovirus has been reported as one of the major causative agents of non-bacterial gastroenteritis in all

Grant sponsor: Ministry of Education, Culture, Sports, Sciences and Technology; Grant sponsor: Ministry of Health, Labor and Welfare; Grant sponsor: Miyakawa Memorial Research Foundation; Grant sponsor: Sumitomo Foundation; Grant sponsor: Mishima-Kaiun Foundation.

*Correspondence to: Hiroshi Ushijima, Department of Developmental Medical Sciences, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail: ushijima@m.u-tokyo.ac.jp

Accepted 10 May 2006

DOI 10.1002/jmv.20696

Published online in Wiley InterScience (www.interscience.wiley.com)

age groups [Inouye et al., 2000; Lopman et al., 2002]. NoV is highly infectious and associated with food-borne and water-borne outbreaks of acute gastroenteritis worldwide in different epidemiologic settings such as hospitals, hotels, schools, cruise ship, and restaurants [Inouye et al., 2000; Billgren et al., 2002; Kageyama et al., 2004]. However, the diarrheal illness due to NoV is usually mild and self-limiting. Global outbreaks of gastroenteritis have been caused previously by different strains of NoV GI and II. Since a study reported by Noel et al. [1999] found the "95/96-US" strain which is grouped into genogoup II genotype 4 (GII/4, known as a Lordsdale cluster) having a global distribution, an unusual increase in the number of NoV outbreaks was reported in Europe and the United States [Lopman et al., 2004; Vipond et al., 2004]. Even NoV infection has a great impact on people in both developing and developed countries; and effective anti-NoV drugs have not been developed. Molecular epidemiology of NoV infection is needed in order to successfully control and prevent illnesses caused by NoV.

The objectives of this study were: to determine the incidence of NoV infections in children with acute gastroenteritis in five different regions of Japan from 1995 to 2001; to characterize NoV detected according to genogroup and genotype; and to describe the genetic diversity among them. Additionally, the age-related distribution and seasonal pattern of NoV infection were determined.

MATERIALS AND METHODS

Fecal Specimens

A total of 3,864 fecal specimens were collected from children with acute gastroenteritis in Sapporo, Tokyo, Maizuru, Osaka, and Saga of Japan from July 1995 to June 2001. The fecal specimens were diluted with distilled water to 10% suspensions, and clarified by centrifugation at 10,000*g* for 10 min. The supernatants were collected and stored at −30°C until use for the detection of NoV.

Extraction of Viral Genome

The viral genomes were extracted from 140 μl of 10% fecal suspensions applying the QIAamp spin-column technique according to the manufacturer's instructions (QIAGEN®, Hilden, Germany).

Reverse Transcription (RT)

For RT, 7.5 μl of extracted viral genome was added with a reagent mixture consisting of 2.05 μl of 5× First strand buffer (Invitrogen, Carlsbad, CA), 0.75 μl of 10 mM dNTPs (Roche, Mannheim, Germany), 0.75 μl of 10 mM DTT (Invitrogen), 0.75 μl (200 U/μl) of superscript reverse transcriptase III (Invitrogen), 0.375 μl (1 μg/μl) of random primer (hexa-deoxyribonucleotide mixture) (Takara, Shiga, Japan), 0.5 μl (33 U/μl) of RNase Inhibitor (Toyobo, Osaka, Japan), and 2.325 μl MilliQ water. The total of the reaction mixture was 15 μl [Yan et al., 2003]. RT step was carried out at 50°C for 1 hr, followed by 99°C for 5 min and then held at 4°C.

Polymerase Chain Reaction (PCR)

Using PCR with specific primers as previously reported resulted in the identification of two genogroups of NoV [Yan et al., 2003]. Two pairs of specific primers G1SKF (CTGCCCGAATTYGTAAATGA) and G1SKR (CCAACCCARCCATTRTACA), and COG2F (CARGAR BCNATGTTYAGRTGGATGAG) and G2SKR (CCRCC NGCATRHCCRTTRTACAT) [where B is C, G, or T; H is A, C, or T; N is any base; R is A or G; and Y is C or T] that amplify capsid gene of NoV were used to detect NoV GI and GII, respectively. These primers were generated specifically for two different sizes of amplicons of 330 bp and 387 bp for NoV GI and NoV GII, respectively. PCR was carried out with 2.5 μl of cDNA in 22.5 μl of the reagent mixture containing 10× Taq DNA polymerase buffer (Promega, Madison, WI), dNTPs (2.5 mM/μl), primers (33 μM), Taq DNA polymerase (5 U/μl) (Promega), and MilliQ water. PCR was performed at 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 7 min, and then held at 4°C.

Electrophoresis

The PCR products were electrophoresed in a 1.5% agarose gel, followed by staining with ethidium bromide for 20 min then visualized under ultraviolet (UV) light, and the results were recorded by photography.

Nucleotide Sequencing and Phylogenetic Analysis

The nucleotide sequences of PCR products (DNA) positive for NoV were determined with the Big-Dye terminator cycle sequencing kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA). Sequence analysis was performed using CLUSTAL X software (Version 1.6). Phylogenetic tree with 100 bootstrap resamples of the nucleotide sequence alignment data sets was generated using the neighbor-joining method with CLUSTAL X. The genetic distance was calculated using Kimura's two-parameter method (PHYLIP). Reference NoV strains and accession numbers used in this study were as follows: Manchester (X86560), Melksham (X81879), Chitta (AB032758), Wortley (AJ277618), Hillington (AJ277607), Toronto (U02030), Lordsdale (X86557), Fayetteville/1998/US (AY113106), Erfurt/546/00/DE (AF42118), M7/99/US (AY130761), Saitama U1 (AB039775), Camberwell (AF145896), Snow (U70059), Arg320 (AF190817), Mexico (U22498), MD145 (AY032605), Mora/97/SE (AY081134), Saitama-KU80aGII/99/JP (AB058582), Bristol (X76716), SaitamaU16 (AB039778), SaitamaU17 (AB039779), WUG1 (AB081723), Chiba (AB022679), Birmingham (AJ277612), and Saitama KU8/99/JP (AB058547).

TABLE I. Distribution of NoV Infection Among Children by Age Group From 1995 to 2001

	0 m	6 m	1 y	2 y	3 y	4 y	5 y	6 y	7 y	8 y	9 y	10 y	ND	Total
1995/1996	1	4	11	7	2	3	1	0	0	0	0	0	6	35
1996/1997	2	6	16	5	2	0	0	0	0	0	0	0	0	31
1997/1998	7	14	27	8	3	1	0	0	0	0	1	1	9	71
1998/1999	3	9	24	9	2	2	3	0	1	0	0	3	4	60
1999/2000	2	14	30	15	9	2	2	3	1	1	2	2	13	96
2000/2001	0	18	21	13	3	2	2	4	3	2	0	2	1	71
Total	15	65	129	57	21	10	8	7	5	3	3	8	33	364

Note: m, month; y, year; ND, not determined.

RESULTS

Epidemiology of NoV Infection

A total of 3,314 fecal specimens collected from children with acute gastroenteritis in Sapporo, Tokyo, Maizuru, Osaka, and Saga of Japan during July 1995 and June 2001 were examined for NoV. In the pediatric population, the lowest age was 0 month and the highest was 10 years. Of 3,314 fecal specimens tested, 364 were detected to be positive for NoV and this represented 11%. Table I showed that the highest NoV infection was in the 1-year old group (35.4%; 129 of 364). The NoV infection was identified among children aged less than 6 months (4.1%; 15 of 364). It was also found that children younger than 3 years had a high rate of NoV infection (73.1%, 266 of 364).

Seasonal Variation of NoV Infection

The NoV detection rate was analyzed between July 1995 and June 2001. Figure 1 shows that NoV was detected continuously for 10 months (September to June). No NoV was found in both July and August. The highest prevalence of NoV infection was in December (41.5%; 151 of 364), followed by January and November with 15.7% (57 of 364) and 13% (47 of 364), respectively. The lowest NoV detection rate was in October (0.3%; 1 of 364).

Distribution of NoV G-Types

The nucleotide sequence of the 5′ ends of the NoV capsid gene was determined by direct sequencing with the amplified fragments. This region has been shown to be suitable for genotyping. All NoV sequences were analyzed by phylogenetics and grouped using the NoV capsid region classification scheme of Kageyama et al. [2004]. In the present study, all of the NoV sequences were classified into two distinct genogroupes I and II and these represented 3% (11 cases) and 97% (353 cases), respectively. The NoV GI sequences clustered into four genotypes (GI/3, GI/4, GI/8, and GI/11), accounting for 27.3% (3 of 11), 54.5% (6 of 11), 9.1% (1 of 11), and 9.1% (1 of 11), respectively. In NoV GII, genotype 4 was dominant every year, from 41.9% (1996–1997) to 80% (1995–1996) followed by genotype 3 as second predominant strain, ranging from 19.1% (1999–2000) to 38% (1997–1998) (Table II). Moreover, many other NoV genotypes including GII/1, GII/2, GII/5, GII/6, GII/9, GII/10, GII/12, GII/13, GII/14, and GII/15 were found

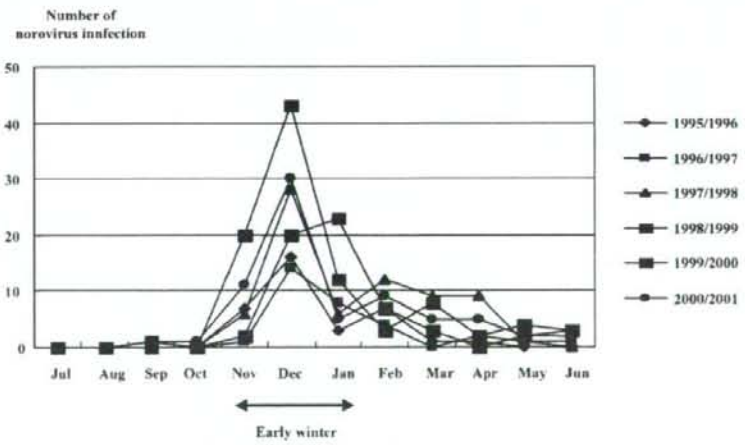

Fig. 1. Seasonal pattern of NoV detected among children with acute gastroenteritis during 6-year survey of NoV infection in Sapporo, Tokyo, Maizuru, Osaka, and Saga of Japan during July 1995 and June 2001. The cold season was also indicated.

TABLE II. Distribution of NoV Genotypes in Five Regions of Japan From 1995 to 2001

				No. (%) of genotypes of GII (1995–1996)						
Regions	Total	GI	GII	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	Rare
Sapporo	73	0	0	0	0	0	0	0	0	0
Tokyo	98	0	11	0	0	8 (72.7)	0	3 (27.3)	0	0
Maizuru	265	0	24	0	0	20 (83.3)	0	3 (12.5)	1 (4.2)	0
Osaka	—	—	—	—	—	—	—	—	—	—
Saga	—	—	—	—	—	—	—	—	—	—
Total	436	0	35	0	0	28 (80)	0	6 (17.1)	1 (2.9)	0
				No. (%) of genotype of GII (1996–1997)						
Regions	Total	GI	GII	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	Rare
Sapporo	50	0	2	0	0	0	0	1 (50)	1 (50)	0
Tokyo	71	0	13	0	10 (76.9)	3 (23.1)	0	0	0	0
Maizuru	239	0	16	1 (6.3)	1 (6.3)	10 (62.5)	1 (6.3)	0	1 (6.3)	G9, G10
Osaka	—	—	—	—	—	—	—	—	—	—
Saga	—	—	—	—	—	—	—	—	—	—
Total	360	0	31	1 (3.2)	11 (35.5)	13 (41.9)	1 (3.2)	1 (3.2)	2 (6.5)	2 (6.5)
				No. (%) of genotypes of GII (1997–1998)						
Regions	Total	GI	GII	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	Rare
Sapporo	62	0	16	0	6 (37.5)	4 (25)	0	5 (31.3)	1 (6.3)	0
Tokyo	93	0	16	0	0	16 (100)	0	0	0	0
Maizuru	249	0	16	0	11 (68.8)	5 (31.3)	0	0	0	0
Osaka	96	0	23	0	10 (43.5)	11 (47.8)	0	1 (4.4)	1 (4.4)	0
Saga	—	—	—	—	—	—	—	—	—	—
Total	500	0	71	0	27 (38.0)	36 (50.7)	0	6 (8.5)	2 (2.8)	0
				No. (%) of genotypes of GII (1998–1999)						
Regions	Total	GI	GII	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	Rare
Sapporo	43	0	2	0	0	2 (100)	0	0	0	0
Tokyo	80	0	7	0	0	5 (71.4)	0	1 (14.3)	1 (14.3)	0
Maizuru	248	0	21	0	3 (14.3)	12 (57.1)	0	6 (28.6)	0	0
Osaka	134	0	23	0	7 (30.4)	12 (52.2)	0	4 (17.4)	0	0
Saga	87	0	7	0	7 (100)	0	0	0	0	0
Total	592	0	60	0	17 (28.3)	31 (51.7)	0	11 (18.3)	1 (1.7)	0
				No. (%) of genotypes of GII (1999–2000)						
Regions	Total	GI	GII	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	Rare
Sapporo	56	0	3	0	3 (100)	0	0	0	0	0
Tokyo	49	GI/4, GI/11	7	0	2 (28.6)	4 (57.1)	1 (14.3)	0	0	0
Maizuru	387	GI/4, GI/3	57	5 (8.8)	5 (8.8)	44 (77.2)	1 (1.8)	1 (1.8)	0	G14
Osaka	121	GI/4, GI/8	14	0	6 (42.9)	7 (50)	0	1 (7.1)	0	0
Saga	153	GI/4	8	0	1 (12.5)	3 (37.5)	0	3 (37.5)	1 (12.5)	0
Total	766	7	89	5 (5.6)	17 (19.1)	58 (65.2)	2 (2.3)	5 (5.6)	1 (1.1)	1 (1.1)
				No. (%) of genotypes of GII (2000–2001)						
Regions	Total	GI	GII	GII/2	GII/3	GII/4	GII/5	GII/6	GII/12	Rare
Sapporo	44	0	5	0	1 (20)	4 (80)	0	0	0	0
Tokyo	37	0	2	0	0	2 (100)	0	0	0	0
Maizuru	365	0	22	0	3 (13.6)	16 (72.7)	0	0	0	G10, G13
Osaka	108	GI/3, GI/4	23	0	7 (30.4)	7 (30.4)	2 (8.7)	4 (17.4)	1 (4.3)	G14, G15
Saga	106	0	15	0	3 (20)	11 (73.3)	0	0	0	G1
Total	660	4	67	0	14 (20.9)	40 (59.7)	2 (3)	4 (6)	1(1.5)	6(9)